The development of the GC-MS analytical method used for the determination of the isotope ratio of linalool in yuzu essential oil from different geographic origins

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Abstract:

A refined analytical method has been developed for the determination of the isotope ratio of oxygenated compounds in essential oils, using high-resolution gas chromatography-mass spectrometry (HRGC-MS), has been developed. Thirty-three samples of yuzu fruits from different production areas in Japan and South Korea were collected and prepared for cold-pressed peel oils. The oils were analysed by HRGC-MS for linalool concentration and isotope ratio based on the peak intensities of $(M+2)^+$, $(M+1)^+$ and M^+ ions. A significantly lower isotope ratio *m/z* 156/154 and *m/z* 155/154 were observed for the yuzu essential oil from Goheung and Kyoto areas. Statistical analysis showed the isotope ratio of linalool to be useful in the discrimination of yuzu essential oils from different geographical origins.

Keywords: ***essential oil, GC-MS, geographical origin, isotope ratio, linalool, yuzu.***

Classification number: ***2.2***

Introduction

Food authenticity is a term which simply refers to whether the food purchased by the consumer matches its description. There are a number of consumer-driven forces for reliable analytical methods to verify the provenance of the food we eat and there is growing enthusiasm amongst consumers for high-quality foods with clear regional identities. It is reasonable to suggest that there should be analytical methods in place that can verify the information provided on origin labels describing the origins of foods.

The stable isotope ratio of constituents is among the many criteria that have been used as discriminators of food authenticity [1, 2]. Naturally abundant isotope ratios of elements exist in a fixed ratio; however, many natural phenomena, classed as physicochemical effects, can also lead to isotope fractionation. The stable isotope ratios of water (oxygen and hydrogen), therefore, can yield unique geographic information [3], primarily because of the predictable spatial variation of precipitation stable isotope ratios across the Earth's surface [4, 5]. This spatial variation in precipitation composition is recorded in plant material since plants take up soil and water, which is derived generally from local precipitation and incorporates the hydrogen and oxygen atoms into the products needed for photosynthesis [6]. This distinction of isotope content can be transferred to plants, creating an isotopic "fingerprint" for geographical characteristics of vegetation. The stable isotope content is determined by isotope ratio mass spectrometry (IRMS) and site-specific natural isotope fractionation determined by nuclear magnetic resonance (SNIF-NMR). These instruments, however, are expensive and rare in normal food and flavour analysis laboratories.

Gas chromatography-mass spectrometry (GC-MS) is an indispensable analytical instrument for analysing food and flavour compounds, especially for analysing volatile compounds. Sawamura, et al. have developed a new analytical method for the differentiation and characterization of citrus essential oils derived from different species and producing areas found on the basis of isotope ratio. The isotope ratio is determined based on the isotope peak and molecular peak of monoterpene hydrocarbons. A combination of the determination of the isotope ratio of multi-components and multivariate analysis results in good discrimination of citrus essential oils of different botanical [7, 8] and geographical origins [9].

In the essential oils of citrus species, oxygenated compounds account for a small but important fraction of keynote compounds. Among the oxygenated compounds, linalool is a main

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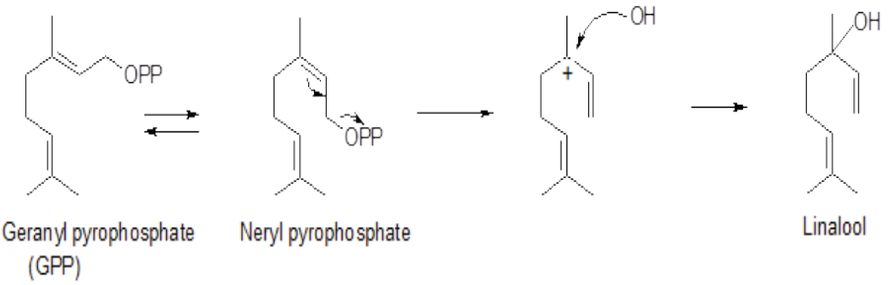

Fig. 1. The biosynthetic pathway of linalool from geranyl pyrophosphate (GPP).

oxygenated compound found in citrus essential oil [10]. Naturally, linalool is synthesised from the universal precursor geranyl pyrophosphate (GPP), catalysed by a membrane-bound enzyme, linalool synthase (Fig. 1) [11]. During this biosynthesis process, water is attached to the carbon frame and therefore, it is expected that it brings in an isotope ratio of hydrogen and oxygen, and thusly, the geographical information of isotope ratio. However, the determination of the isotope ratio of oxygenated compounds via current methods is challenging due to the weak signal strength of the isotope and molecular peaks. In this study, we designed a new approach to the determination of the isotope ratio of oxygen in order to find an additional analytical parameter to be used for the discrimination of essential oils and fruits from different origins. Yuzu (*Citrus junos* Tanaka), an important sour citrus fruit in Japan, and is especially found in Kochi prefecture, was investigated. Yuzu essential oils were derived from different producing areas in Japan and Korea, and were analysed for their isotope ratios through means of HRGC-MS.

Materials and methods

Materials

Authentic linalool was obtained from Tokyo Kasei Kogyo Co. Ltd. Standard solutions of linalool were prepared at different concentrations with purified acetone (purity ≥ 99.8%, Kanto Chemical Co., Inc.). 33 samples of yuzu were collected from Japan and South Korea (Fig. 2). The essential oil was prepared using the cold-pressing method to isolate citrus essential oils [12].

Fig. 2. Yuzu Sampling in Japan and Korea.

GC-MS

Analysis was carried out using a GC-6890N instrument (Agilent Technologies) coupled with a JMS-Q1000 GTA mass spectrometer (Jeol Datum) at an MS ionization energy of 70 eV; detector voltage, 1000 V; ionization current, 100 mA; and ion source temperature of 250°C. The GC column was a DB-Wax fused-silica capillary type (60 m × 0.25 mm i.d., 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). To determine the linalool peak relative percentage, scan mode was used. An oil sample of 1 µl, which had been diluted with acetone (1:5), was automatically injected at a split ratio of 1:100. The column temperature was programmed from 70°C (2-min hold) to 100°C at a rate of 2°C/min and then heated to 230°C (held for 15 min) for sterilisation at the end of each run. The injector temperature was 250°C, and helium was used as the carrier gas at a flow rate of 0.8 ml/min.

Determination of isotope ratio of linalool by GC-MS

Isotope ratio is defined as the ratio of the concentration of ions in an isotope and molecular peak, directly observed by the signal area of ions' peak in the mass spectrometry (MS). The selected ion monitoring mode (SIM) was employed to enable the sensitivity of MS analyses. Three ions of linalool were determined for ion concentrations: the molecular ion (M^+): *m/z* 154, the isotope ion $(M+1)^+$: *m/z* 155 and $(M+2)^+$: *m/z* 156. An optimised condition of MS was developed because the signal strength of an isotope peak *m/z* 156 is difficult to observe under normal analytical conditions. An oil sample of 1 µl, which had been diluted with acetone (1:5), was automatically injected at a split ratio of 1:10. The ion source temperature was 150°C; ionisation current, 200 mA; the detector voltage, 1500 V; and the scanning rate of *m/z* 154 ion and *m/z* 155 ion was 50 cycles per second, while that of *m/z* 156 was 900 cycles per second.

The isotope ratio (Ir) was calculated using the following equation:

$$\text{Ir}\ (\%) = \frac{\text{Intensity of isotope peak}}{\text{Intensity of molecular peak}} \text{x}100$$

where: the isotope peaks were *m/z* 155 and *m/z* 156, respectively; and the molecular peak was *m/z* 154. Each value is the mean of replicate measurements of isotope ratio values.

Statistical analysis

All measurements were carried out in triplicate so that an average value and standard deviation of the isotope value could be calculated to evaluate the repeatability of the method. Analysis of variance (one-way ANOVA) was conducted to differentiate samples by means of the isotope ratio values. All statistical analyses were done using SPSS software for Windows (version 11.5, SPSS, Chicago, 2002).

Results and discussions

Accuracy of the isotope ratio by ordinary GC-MS

In principle, it is possible to obtain the isotope ratio from MS data [13]. The isotope peak contains the total isotopic abundance in the molecule. Authors have previously shown, in fact, a practical use for the isotope ratio of monoterpene hydrocarbon from mass spectrometry [7-9, 14]. However, the determination of isotope ratio in oxygenated compounds was difficult since the intensity of each molecular ion peak is not very strong after undergoing the fragmentation due to ionisation process of MS. In addition, the isotope peak (M+1) is approximately 10%, whereas the isotope peak (M+2) is approximately 1%, respectively, and of those observations, the molecular ion peak in the case of linalool in citrus essential oils is seen. Therefore, several experimental conditions have been developed to achieve a sensitivity of the analytical method for determining isotope ratio value. By choosing the softer ionisation energy (50 eV), suitable detector voltage, and scanning rate of the monitored ion, the desired ion peaks were enabled since optimisation of the analysis device and the analysis condition was attempted in the actual experiment. The mass peaks observed in the mass spectrum are *m/z* 154 $(M)^+$, *m/z* 155 $(M+1)^+$ and *m/z* 156 $(M+2)^+$. When taking the ratio of those peak intensities, the isotope ratio *m/z* 155/154 accordingly gives the total ratios of $^{13}C/^{12}C$, $^{2}H/^{1}H$, and also $^{17}O/^{16}O$; and the isotope ratio *m/z* 156/154 gives information on $^{18}O/^{16}O$ and $^{13}C/^{12}C$.

The repeatability of the Ir values for linalool was examined using different linalool solutions. The concentrations of standard linalool solutions were 5 to 30 mg/g (w/w). Data is shown in Table 1, the isotope ratio *m/z* 155/154 varied from 11.50 to 11.60% whereas the isotope ratio value *m/z* 156/154 was 1.00%. These Ir values varied within a narrow range and in accordance with theoretical calculations from natural abundances of isotopes of carbon, hydrogen, and oxygen. The results obtained from the authentic linalool solution show that the reproducibility for determining the peaks of the molecular ion and its isotope using ordinary GC-MS is satisfactory and is applicable for practical use.

Table 1. The isotope ratio (%) of authentic linalool solutions at different concentrations.

Linalool concentration mg/g (w/w)	*m/z* 156/154		*m/z* 155/154	
	Mean[1]	*SD*	*Mean*[1]	*SD*
5	1.10	0.02	11.64	0.06
10	1.06	0.05	11.59	0.05
15	1.11	0.03	11.52	0.04
20	1.09	0.02	11.50	0.02
25	1.11	0.03	11.54	0.01
30	1.09	0.01	11.55	0.01

[1]n=5.

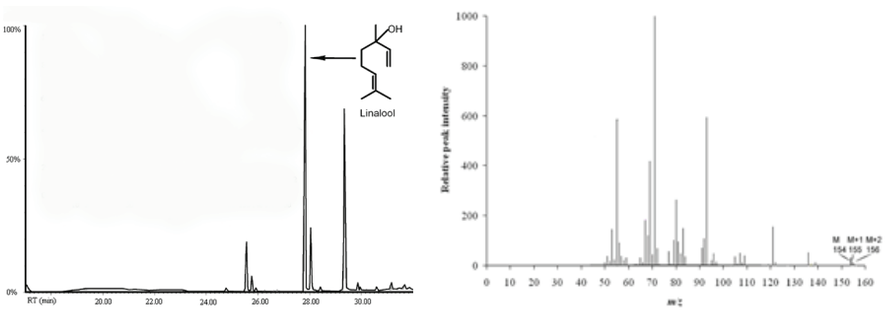

Fig. 3. GC chromatogram of yuzu essential oil by capillary GC-MS and MS spectrum of linalool.

The repeatability, evaluated on relative chromatographic peak areas of the standard linalool solution using ten replicates that were analysed in the same day, was 3.1%. The reproducibility, calculated using five replicates of the same solution analysed in different days, was 8.8%. The limits of detection and the limits of quantitation were calculated from the concentration that would give up to three and ten times, respectively, and were also reported. The limit of detection (LOD) and limit of quantification (LOQ) were determined from a series of low-concentration measurements of the authentic linalool calibration solutions. The data processing was proved under SIM mode to increase the specificity and sensitivity of the measurement. However, since the isotope peak of linalool was small compared to the molecular peak, we need to determine the isotope peaks *m/z* 155 (M^+) and *m/z* 156 (M^{2+}) so the detection limit was dependent on the appearance of those isotope peaks. The resulting LOD and LOQ were 10 and 35 mg/kg, respectively.

The isotope ratios of linalool in yuzu essential oils from different producing areas

A proper separation of linalool from other volatile compounds of the essential oil is a prerequisite to the determination of isotope ratio value. The linalool fraction was well separated from the other volatile compounds in the yuzu essential oil by employing appropriate column and column temperature program. The investigated compound

Table 2. Relative peak area percentage (%) and isotope ratio of linalool from yuzu essential oils.

No	Sample[1]	Relative peak area (%)	Isotope ratio (%)			
			m/z 155/154		*m/z* 156/154	
			Mean[2]	*SD*	*Mean*[2]	*SD*
1	EH1	3.04	14.35	0.49	1.02	0.01
2	EH2	2.19	12.91	0.06	0.91	0.00
3	EH3	1.97	12.38	0.09	0.88	0.00
4	OT1	2.95	14.46	0.63	1.03	0.02
5	OT2	2.31	13.24	0.09	0.95	0.01
6	OT3	2.24	13.28	0.13	0.94	0.02
7	WK1	2.90	14.78	0.17	1.04	0.02
8	WK2	2.36	13.54	0.03	0.96	0.01
9	WK3	2.61	13.83	0.13	0.98	0.01
10	TK1	2.71	14.07	0.60	1.00	0.02
11	TK2	2.59	13.86	0.14	0.98	0.01
12	TK3	2.58	13.84	0.09	0.98	0.01
13	KC1	2.98	13.05	0.69	0.88	0.18
14	KC2	2.14	12.59	0.35	0.90	0.01
15	KC3	1.72	11.77	0.19	0.83	0.00
16	KC4	1.25	10.84	0.06	0.91	0.17
17	KC5	1.28	10.89	0.12	0.92	0.16
18	KC6	1.27	11.45	0.05	1.10	0.01
19	KC7	1.31	11.10	0.66	0.94	0.03
20	KC8	1.28	10.67	0.05	0.97	0.05
21	KC9	1.24	10.71	0.09	0.97	0.04
22	KC10	1.28	10.76	0.20	0.92	0.05
23	KC11	1.31	10.76	0.10	0.97	0.03
24	KC12	1.28	10.69	0.09	0.96	0.02
25	KC13	1.18	10.78	0.06	1.05	0.07
26	KC14	1.32	11.13	0.73	0.96	0.01
27	KC15	1.35	10.69	0.17	0.94	0.03
28	KC16	1.40	10.64	0.02	0.96	0.04
29	KC17	1.38	11.08	0.03	1.00	0.03
30	KC18	1.46	11.04	0.61	0.94	0.00
31	KC19	1.46	10.93	0.04	0.99	0.01
32	KYO	2.55	11.61	0.02	0.93	0.04
33	GOH	3.58	11.53	0.04	0.91	0.06

[1]Abbreviated name of samples: KC: Kochi; EH: Ehime; TK: Tokushima; OT: Oita; WK: Wakayama; KYO: Kyoto; GOH: Goheung (Korea).
[2]n = 3.

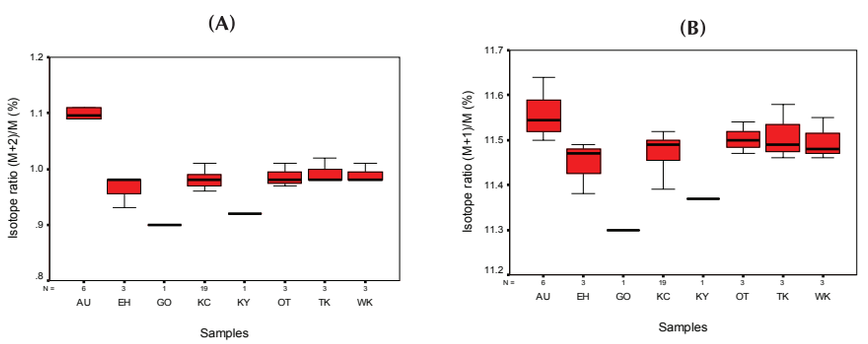

Fig. 4. Boxplot of isotope ratio (M+2)/M (A) and (M+1)/M (B) of linalool from authentic chemicals and yuzu essential oils from different producing areas. Samples from different areas are marked as Authentic chemicals, AU; Ehime, EH; Goheung, GO; Kochi, KC; Kyoto, KY; Oita, OT; Tokushima, TK; Wakayama, WK.

was best separated on DB-Wax column with high polarity. Fig. 3 shows the linalool peak in the GC chromatogram of yuzu essential oil under the actual experimental condition.

Relative peak area and isotope ratio of linalool from 33 yuzu essential oils are shown in Table 2. The isotope ratio *m/z* 156/154 of linalool in standard solution and in yuzu CPO from different producing areas is plotted in the boxplot (Fig. 4). It is obvious that the standard linalool has a significantly higher isotope ratio value (mean Ir=1.10% with SD=0.02%). The isotope ratio values of linalool from yuzu samples which were grown in Kochi (KC), Ehime (EH), Oita (OT), Tokushima (TK), and Wakayama (WK), were not different from one another. The average isotope ratio value ranged from 0.96 to 1.02% with a standard deviation less than 0.6%. The isotope ratio of linalool in GOH and KYO samples, on the other hand, significantly lower than the other yuzu samples. Ir values were 0.89 and 0.91% for GOH and KYO, respectively. These differences can be explained by the fact that authentic linalool from which the compound was isolated and/or synthesised was obtained from different sources.

Conclusions

In conclusion, to the best of our knowledge, the isotope ratio of oxygenated compounds in yuzu essential oils by HRGC-MS is reported here for the first time. The analytical method was developed and optimised for the observation of linalool isotope peaks and molecular peaks and showed high repeatability. The isotope ratio of linalool varied considerably among the samples from different regions, but could not readily be differentiated on the basis of isotope ratio value alone. The isotope ratio depends on the genealogy and geographical factors of the plant [6]. However, in this study, when all the

samples were obtained from the same botanical origin, the effect of genealogy may be eliminated. Thus the isotope ratio reflexes the effect of producing area. The significant lower isotope ratio of the samples from higher latitude is in agreement of lower isotope ratio in the region of higher latitude. This finding suggests a value for the application of GC-MS to authenticity control by means of component isotope ratio.

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REFERENCES

[1] A. Rossmann (2001), "Determination of stable isotope ratios in food analysis", *Food Reviews International*, **17**, pp.347-381.

[2] Christopher N. Rhodes, Janice H. Lofthouse, Simon Hird, Paul Rose, Paul Reece, Julie Christy, Roy Macarthur, Paul A. Brereton (2010), "The use of stable carbon isotopes to authenticate claims that poultry have been corn-fed", *Food Chemistry*, **118,** pp.927-932.

[3] S. Asche, A.L. Michaud, J.T. Brenna (2003), "Sourcing organic compounds based on natural isotopic variations measured by high precision isotope ratio mass spectrometry", *Current Organic Chemistry*, **7,** pp.1527-1543.

[4] Y. Yuntseover, and J.R. Gat (1981), *Atmostpheric water*, IAEA technical report series 210-stable isotope hydrology, pp.103-142.

[5] H. Craig (1961), "Isotopic Variations in Meteoric Waters", *Science*, **133(3465),** pp.1702-1703.

[6] T.E. Dawson, S. Mambelli, A.H. Plamboeck, P.H. Templer, K.P. Tu (2002), "Stable isotopes in plant ecology", *Annual Review of Ecology and Systematics*, **33(1),** pp.507-559.

[7] N.T. Thao, T. Kashiwagi, M. Sawamura (2007), "Characterization by GC-MS of Vietnamese Citrus Species and Hybrids Based on the Isotope Ratio of Monoterpene Hydrocarbons", *Biosci. Biotechnol. Biochem.*, **71(9),** pp.2155-2161.

[8] A. Satake, A. Une, T. Ueno, H. Ukeda, M. Sawamura (2003), "Isotope ratio analysis by HRGC-MS of monoterpene hydrocarbons from citrus essential oils", *Biosci. Biotechnol. Biochem.*, **67(3),** pp.599-604.

[9] M. Sawamura, T. Ito, A. Une, H. Ukeda, Y. Yamasaki (2001), "Isotope ratio by HRGC-MS of citrus *Junos tanaka* (yuzu) essential oils: *m/z* 137/136 of terpene hydrocarbons", *Biosci. Biotechnol. Biochem.*, **65(12),** pp.2622-2629.

[10] M. Sawamura (2000), "Volatile components of essential oils of the *Citrus* genus", *Agricultural & Food Chem.*, **4(1),** pp.131-164.

[11] M. Sawamura (1994), "Citrus flavour", *Kagaku to seibutsu*, **32,** pp.114-119.

[12] S.M. Njoroge, H. Koaze, P.N. Karanja, M. Sawamura (2005), "Volatile constituents of redblush grapefruit (*Citrus paradisi*) and pummelo (*Citrus grandis*) peel essential oils from Kenya", *J. Agric. Food Chem.*, **53(25),** pp.9790-9794.

[13] F.W. McLafferty, and F. Turecek (1993), *Interpretation of mass spectra*, University Science Books, p.371.

[14] A. Satake, K. Furukawa, T. Ueno, H. Ukeda, M. Sawamura (2004), "MS fragment isotope ratio analysis for evaluation of citrus essential oils by HRGC-MS", *Biosci. Biotechnol. Biochem.*, **68(2),** pp.312-316.